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ISOLATION AND CHARACTERIZATION OF HEAT-SHOCK PROTEIN 90 (HSP90) SPECIFIC PROMOTER OF *Cryptocoryne ciliata*

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ABSTRACT

Plants are equipped with various mechanisms for survival in extreme environments. Their ability to grow and adapt to extreme conditions such as high salinity, osmotic stress and ion toxicity is amazing. Heat-shock protein, particularly Hsp90, is one of the important parts of the chaperone machinery that involves in the protection of the structure and functions of cells and has a significant task in maintaining the cellular homeostasis. To further understand the functions of Hsp90 protein, a specific promoter for the *CcHsp90-2* gene that encoded 700 amino acids (GenBank accession number: JN120021) was isolated from *Cryptocoryne ciliata* using the genome walker approach and later characterized. Based on the functional motif of the sequence (319 bp), the promoter consists of 13 *cis*-elements and stress factors such as light responsiveness, methyl jasmonate (MeJA) responsiveness, jasmonate-responsiveness (JERE) and many more. This finding gives further understanding on acquired stress tolerance of Hsp90 such as communication of specific Hsp/chaperones in a crowded cellular environment, the specific regulation of Hsp/chaperone molecules, participation in stress sensing, signal transduction and transcription activation of the stress gene.

Key words: Heat-shock protein 90, Chaperones, *Cryptocoryne ciliata*

INTRODUCTION

Cryptocoryne ciliata, also known as "keladi payau" in Malaysia, is an aquatic monocot plant, a member of the aroid family (Araceae). This plant, which is a native tropical halophyte extending from India to Papua New Guinea, lives mostly in streams, rivers, lowland forest areas, seasonally inundated forest pools and also on river banks submerged only at high water. *C. ciliata* has the ability to adapt in brackish water (Simon *et al*., 2008) and is continuously exposed to the extreme environment that generally causes osmotic stress and ion toxicity for most terrestrial plants (Nakayama *et al*., 2005).

In nature, plants are routinely exposed or subjected to a combination of different types of environmental stresses. In response, plants have developed a wide range of adaptation strategies to ensure their survival. However, responses to a specific stress can vary with the plant genotype. Environmental stresses affect different cellular processes such as photosynthesis, growth, carbohydrate and lipid metabolisms, protein synthesis, osmotic pressure and gene expression depending on the type of stress (Legay *et al*., 2009). Plants need to accumulate many metabolites known as "compatible solutes" by changing the levels of hormones, ion and other molecules such as abscisic acid, calcium, jasmonic acid and inositol in response to stresses (Taji *et al*., 2004). These compounds take part in the regulation of stress responsive genes through specific transcription factors and phosphorylation cascades which turn on various cellular components such as polyamine, carbohydrates, amino acids, late embryogenesis abundant (LEA) proteins, ions, heat-shock proteins (Hsps) and water channels (Legay *et al*., 2009). Many of these compounds are involved in the adaptive mechanisms, for example osmoprotection, protein folding, turgor maintenance and detoxification. Heat-shock proteins 90 (Hsp90) have been identified in plant homeostasis and play a crucial role in protecting against stress by re-

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establishing normal protein conformation and thus cellular homeostasis (Wang *et al*., 2004).

Hsp90 is one of the most abundant proteins expressed in cells, about 1 to 2% of the total cellular proteins under normal conditions. It is found in bacteria and eukaryotes, but apparently absent in Archaea (Chen *et al*., 2006). This protein is encoded by a multi-gene family with structurally related proteins from 80 to 108 kDa molecular mass (Reddy *et al*., 2011). However, there are slight differences, depending on the organisms, in their relative molecular masses which are represented by different names (e.g. Hsp80, Hsp81, Hsp82, Hsp83, Hsp84, Hsp90, etc.). In the present study, the common term Hsp90 is used to represent all of these protein orthologues and paralogues. Apart from acting as a "housekeeper", Hsp90 also plays a major role in the managing of protein folding (Young *et al*., 2001) and protecting the cells from stresses. It also plays a key role in signal transduction networks, cell-cycle control, protein degradation and protein trafficking (Krishna and Gloor, 2001). This overall molecular process is controlled by a specific promoter that contains specific elements, that initiates transcription of a particular gene. In this study, we isolated and characterized the specific Hsp90 promoter of *C. ciliata* to predict the actual functions and discover the *cis*-elements that control the expression of *CcHsp90-2* gene.

MATERIALS AND METHODS

Isolation of *CcHsp90-2* gene specific promoter

The specific promoter for the *Cryptocoryne ciliata* heat-shock protein 90 (*CcHsp90-2*) gene was isolated using the GenomeWalker™ Universal Kit (Clontech, USA). The genomic DNA of *C. ciliata* was extracted using the Wizard® Genomic DNA Purification Kit (Promega, USA) and digested with four different restriction endonucleases (*Dra*I, *Eco*RV, *Sma*I and *Stu*I) before it was ligated to the long suppression adapters using the T4 ligase enzyme. The desired genomic region was amplified with a specific primer (GWAP1, 5'-GTA ATA CGA CTC ACT ATA GGG-3') to the outer part of the suppression adapter and a gene-specific reverse primer (GWR1, 5'-CCC GAA GGA AGA TTT CCT TG-3') designed from the *CcHsp90-2* gene sequence. The PCR program Mastercycler Gradient, (Eppendorf, Germany) was applied as follows: initial denaturation at 98ºC for 30 sec; 7 cycles denaturation at 98ºC for 15 sec, annealing at 65ºC for 30 sec and extension at 72ºC for 2 min; 32 cycles denaturation at 98ºC for 15 sec, annealing at 60ºC for 30 sec and extension at 72ºC for 2 min; final extension at 72ºC for 10 min. Then, 1 µl of PCR product was re-amplified using the GWAP2 forward primer (5'-ACT ATA GGG CAC GCG TGG T-3') and GWR2 reverse primer (5'-GAG TAG AAG GTG TTG ATG ATG) in 50 µl of PCR reaction, and the PCR program applied was as follows: initial denaturation at 98ºC for 30 sec; 5 cycles at 98ºC for 15 sec (denaturation), 65ºC for 30 sec (annealing) and 72ºC for 2 min (extension); 20 cycles at 98ºC for 15 sec (denaturation), 60ºC for 30 sec (annealing) and 72ºC for 2 min (extension); final extension at 72ºC for 10 min. The PCR products were then electrophoresed in 1.5% agarose gel added with TAE buffer (pH 8.0) and 10 µg/µl of ethidium bromide, at 80 volts for 45 min and subsequently analysed. The PCR band was purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA), ligated into pGEM-T vector (Promega, USA) before it was cloned into *Escherichia coli* DH5α competent cells and sequenced.

Characterization of *CcHsp90-2* gene specific promoter

The sequencing results were analysed using; Basic Local Alignment Search Tool (BLAST), http://blast.ncbi.nlm.nih.gov/Blast.cgi (Altschul *et al*., 1990); Multiple sequence alignment, https://www.ebi.ac.uk/Tools/msa/clustalw2/ (Larkin *et al*., 2007); Plant *Cis*-Acting Regulatory Element (PlantCARE), http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ (Lescot *et al*., 2002).

RESULTS AND DISCUSSION

Analysis of gel electrophoresis showed two clear DNA bands (Fig. 1) with an approximate size of 500 bp (genome digested with *Dra*I) and 400 bp (genome digested with *Eco*RV), which were successfully cloned and sequenced. Based on the sequenced data results, genome digested with *Dra*I produced a 468 bp fragment which showed positive hits to Hsp90 gene promoter and registered in the GenBank database (accession number: KJ801398) (Fig. 2). Multiple sequence alignment using the ClustalW2 software shows the 100% similarity of overlapping region *CcHsp90-2* gene with isolated fragment (Fig. 3). This result showed that the designed primers amplified the target sequences correctly. Analysis of *CcHsp90-2* gene specific promoter sequence using PlantCARE showed that there are 13 functional motifs in this fragment (Table 1). The existence of TATA-box motif that which is the core promoter sequence suggested that the isolated fragment is a specific promoter for the *CcHsp90-2* gene. The TATA-box is a binding site of either general transcription factors or histones (the binding of a transcription factor blocks the binding of a histone and vice versa) and is involved in the process of transcription by RNA polymerase (Yang *et al*.,

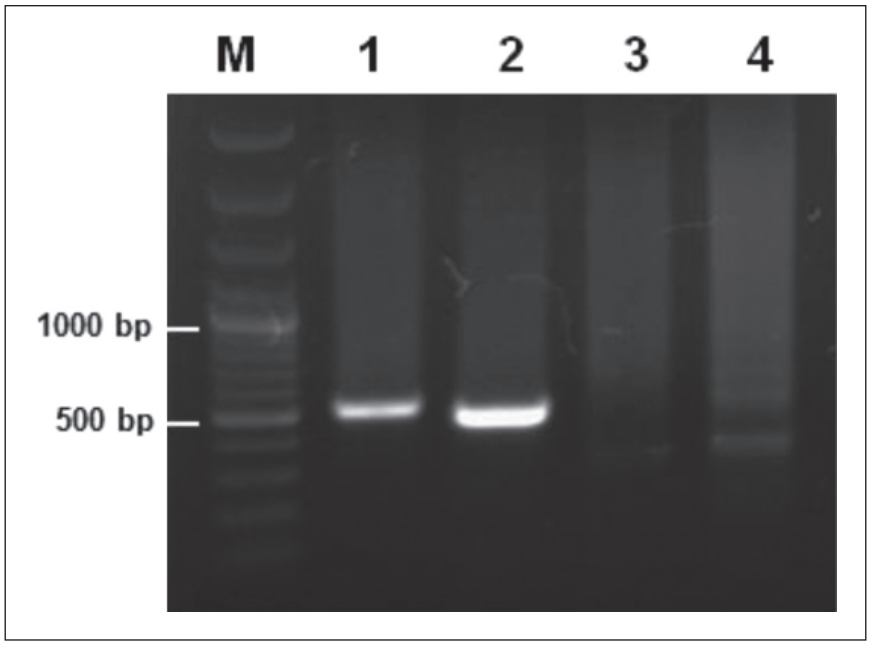

Fig. 1. The PCR amplification of genome walking product using digested *C. ciliata* genome of four different restriction enzymes; *Dra*I (lane 1), *Eco*RV (lane 2), *Sma*I (lane 3) *Stu*I (lane 4). M lane, 100 bp plus DNA ladder (Fermentas, Lithuania).

5′-AAGTTTTTTTTTACCTTGTATTTCTCTGTTCACTTATCATACAAACCCGTACAGAAAGT
TTTCTTAAAAGAGCGTATCGTTATTTTTATTTTTTGAAGCGCAATTAATTTTTAAGCGAA
AGGTAAATGTAAAAAAAAGGGAAAAGATCGTCCGCGACCGTGACGTCAGCTTTAACATAT
CCGCGAAGGATCTAGAAGGTAGAGGTCTGGTTAAACTAACCCGCGAAACTTGGGGCTCGG
AAGGATCTAGAAGGGAAGAGTGGCCGGCGGTCTGCCCTCTTTTATAGGCCACTTCTCTCC
TCCTCCTCCTTGGTCCTTCTAAACCCTAACGGGCGCCGTTTCTGTGGTTGTGCGTCGGTT
TCGGCTTCGTCTCGCAGGCCGCAGGGCGGAGGATGGCAGCGGAGACGGAGACCTTCGCCT
TCCAGGCGGAGATCAACCAGCTTCTCAGTCTCATCATCAACACCTTCTACTC-3′

Fig. 2. DNA sequence (5’ to 3’) of the genome walking product using genome digested with *Dra*I (471 bp) containing; 319 bp of *CcHsp90-2* gene specific promoter, 72 bp of *CcHsp90-2* 5’-untranslated region (underline) and 80 bp of partial *CcHsp90-2* gene sequence (bold).

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KJ801398   AAACCCTAACGGGCGCCGTTTCTGTGGTTGTGCGTCGGTTTCGGCTTCGTCTCGCAGGCC 60
CcHsp90-2  AAACCCTAACGGGCGCCGTTTCTGTGGTTGTGCGTCGGTTTCGGCTTCGTCTCGCAGGCC 60
           ************************************************************

KJ801398   GCAGGGCGGAGGATGGCAGCGGAGACGGAGACCTTCGCCTTCCAGGCGGAGATCAACCAG 120
CcHsp90-2  GCAGGGCGGAGGATGGCAGCGGAGACGGAGACCTTCGCCTTCCAGGCGGAGATCAACCAG 120
           ************************************************************

KJ801398   CTTCTCAGTCTCATCATCAACACCTTCTACTC 152
CcHsp90-2  CTTCTCAGTCTCATCATCAACACCTTCTACTC 152
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Fig. 3. Multiple sequence alignment of *CcHsp90-2* gene with KJ801398 fragment showing 100% similarity. Numbers at the end of each sequence rows indicate nucleotide counts and asterisks indicate similarity.

Table 1. List of functional motifs found in *CcHsp90-2* gene specific promoter

MOTIF	SEQUENCE	FUNCTION
- AAGAA-motif	GTAAAGAAA	- unknown
- Box 4	ATTAAT	- part of conserved DNA module involved in light responsiveness
- C-box	CTGACGTCAG	- *cis*-acting regulatory element involved in light responsiveness
- CAAT-box	CAATT	- common *cis*-acting element in promoter and enhancer regions
- CAT-box	GCCACT	- *cis*-acting regulatory element related to meristem expression
- CGTCA-motif	CGTCA	- *cis*-acting regulatory element involved in MeJA-responsiveness
- GT1-motif	GGTTAA	- light responsive element
- JERE	AGACCGCC	- Jasmonate-responsiveness element
- TATA-box	TATA, TTTTA, TATAA, TATAAAA, ccTATAAAaa, TAAAAATAA and TATAAA	- core promoter element around-30 bp of transcription start
- TC- rich repeats	GTTTTCTTAC	- *cis*-acting regulatory element involved in defence and stress responsiveness
- TGACG-motif	TGACG	- *cis*-acting regulatory element involved in the MeJA-responsiveness
- As1	TCACGTCA	- *cis*-acting regulatory element involved in root-specific expression
- Chs-Unit 1 m1	ACCTAACCCGC	- part of a light responsive element

2007). This motif is usually located 30 bp upstream from the transcription start site. Environmental stress evokes multiple responses of molecular, biochemical and physiological events. Stress is deleterious to the structures and functions of many structural proteins and enzymes. Consequently, proteins should be maintained from denaturation, aggregation and misfolding that are particularly important for cell survival under stress conditions (Efeoğlu, 2009). According to Liu *et al*. (2006), the expression of Hsp90 responds not only to the high temperature but more towards environmental stresses such as salinity, drought, cold and pH changes. This statement is supported by discovery of light responsiveness (C-box, chs-Unit 1 m1, and GT1 motif), defence and stress responsiveness (TC- rich repeats) and methyl jasmonate-responsiveness (TGACG-motif and JERE) *cis*-acting elements in the *CcHsp90-2* gene specific promoter. Moreover, As-motif and CAT-box elements, which control the expression of Hsp90 protein at specific plant organ such roots and meristem were also found in this gene promoter.

CONCLUSION

The putative fragment of *CcHsp90-2* gene promoter was isolated and characterized to predict the actual functions and *cis*-elements that control the expression of the gene. This study provided important information for understanding the roles and functions of Hsp90 protein in adaptation of *C. ciliata* (halophyte plant) to adverse environmental stress which is a major factor that limits crop productivity all over the world.

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